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REVIEW

Lysophosphatidic acid in atherosclerotic diseases

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Lysophosphatidic acid (LPA) is a potent bioactive phospholipid. As many other biological active lipids, LPA is an autacoid: it is formed locally on demand, and it acts locally near its site of synthesis. LPA has a plethora of biological activities on blood cells (platelets, monocytes) and cells of the vessel wall (endothelial cells, smooth muscle cells, macrophages) that are all key players in atherosclerotic and atherothrombotic processes. The specific cellular actions of LPA are determined by its multifaceted molecular structures, the expression of multiple G-protein coupled LPA receptors at the cell surface and their diverse coupling to intracellular signalling pathways. Numerous studies have now shown that LPA has thrombogenic and atherogenic actions. Here, we aim to provide a comprehensive, yet concise, thoughtful and critical review of this exciting research area and to pinpoint potential pharmacological targets for inhibiting thrombogenic and atherogenic activities of LPA. We hope that the review will serve to accelerate knowledge of basic and clinical science, and to foster drug development in the field of LPA and atherosclerotic/atherothrombotic diseases.

Abbreviations

APT1, acyl-protein thioesterase 1; ATX, autotaxin; CCL2, chemokine ligand 2; cPA, cyclic phosphatidic acid; CREB, cAMP response element-binding; Edg, endothelial differentiation gene; Egr, early growth response gene; ESI, electrospray ionization; ICAM-1, intracellular adhesion molecule-1; LC, liquid chromatography; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPL, lysophospholipids; LPP, lipid phosphate phosphatase; Lp-PLA$_2$, lipoprotein-associated PLA$_2$; LPS, lysophosphatidylserine; LYPLA-I, lysophospholipase A-I; lysoPLD, lysophospholipase D; MCP-1, monocyte chemotactic protein-1; MLC, myosin light chain; MM–LDL, minimally modified LDL; mox-LDL, mildly oxidized LDL; MS, mass spectrometry; NVAF, nonvalvular atrial fibrillation; PA, phosphatidic acid; PAF, platelet-activating factor; PAK, p21-activated kinase; PC, phosphatidylcholine; PI3K, phosphoinositide 3-kinase; PLA, phospholipase A; ox-LDL, oxidized LDL; SBI, silent brain infarction; S1P, sphingosine 1-phosphate; SMD, somatomedin domain; SPCs, smooth muscle progenitor cells; sPLA2, secretory PLA$_2$; SRF, serum response factor; TF, tissue factor; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular smooth muscle cells

Introduction

Atherosclerosis is a slowly progressing, multifocal, chronic arterial disease that is characterized by inflammatory and regenerative processes, which lead to matrix remodelling and large lipid deposits. The retention of low-density lipoproteins (LDL) and activation of endothelial cells initiate atherosclerotic lesion formation in the inner layer (intima) of medium- and large-sized arteries, such as coronary arteries and cerebral arteries, predominantly at predilection sites, where the laminar blood flow is disturbed (Libby *et al.*, 2011; Weber and Noels, 2011). In early lesions, monocytes are recruited to the arterial wall, where they engulf lipids and transform into foam cells. In the advanced stages of lesion formation, a fibroatheroma develops in the intima that is characterized by the extracellular accumulation of lipids and a fibrous cap

consisting of vascular smooth muscle cells (VSMCs) and extracellular matrix proteins. Although patients with atherosclerosis may not develop clinical symptoms, the narrowing of the arterial lumen may compromise the oxygen supply and result in ischaemia of the tissues that are supplied by the affected artery, such as the myocardium or the brain. Furthermore, acute thrombotic occlusion of the arterial lumen can occur following erosion or rupture of atherosclerotic plaques, which leads to platelet activation and fibrin formation. These acute atherothrombotic events are potentially life-threatening sequelae of atherosclerosis as they may lead to myocardial infarction or stroke (Fuster *et al.*, 2005; Thim *et al.*, 2008). Percutaneous interventions, such as coronary stent implantation, can reopen the occluded or narrowed arteries and limit ischaemic symptoms, and are often life-saving. However, such interventions can result in a re-narrowing of the target vessel (restenosis) due to the accumulation of SMCs in the neointima.

Lysophosphatidic acid (LPA) is a phospholipid that mediates a plethora of activities in blood cells and cells of the vessel wall (Tigyi, 2001; Siess, 2002; Siess and Tigyi, 2004; Smyth *et al.*, 2008). Similar to other lipid mediators such as prostaglandins and leukotrienes, LPA acts as an autacoid. After local formation in response to danger signals such as those initiated by vascular injury or inflammation LPA rapidly activates cells in the immediate vicinity (Tokumura, 1995). The action of LPA is mediated by its binding to and activation of surface GPCRs, including the Edg family of LPA receptors LPA_{1-3}, LPA_4 (GPR23), LPA_5 (GPR92), and LPA_6 (P2Y5), the latter three belonging to the purinoceptor cluster (Choi *et al.*, 2010; Chun *et al.*, 2010; Tigyi, 2010). Currently putative LPA GPCRs are GPR87, P2Y10 and GPR35; hence, there are in total nine LPA receptors (Choi *et al.*, 2010; Chun *et al.*, 2010; Tigyi, 2010). In addition to GPCRs, some of the effects of LPA appear to be mediated by the activation of the nuclear receptor PPARγ, specifically in macrophages and VSMCs (McIntyre *et al.*, 2003; Zhang *et al.*, 2004).

Our review is focused on the role of LPA and its receptors in atherosclerosis and cardiovascular diseases. Since the seminal observations that LPA is generated during mild oxidation of LDL and accumulates in the lipid core of human atherosclerotic plaques linking, for the first time, LPA to atherosclerosis (Siess *et al.*, 1999), the area has evolved dramatically. We aim to cover this research field in the present review. General aspects of LPA receptor functions and their coupling to intracellular signalling pathways (Choi *et al.*, 2010; Tigyi, 2010; Yanagida and Ishii, 2011), of intra- and extracellular LPA formation and metabolism (Nakanaga *et al.*, 2010; Samadi *et al.*, 2011; van Meeteren and Moolenaar, 2007), and of the role of LPA in cardiovascular physiology (Smyth *et al.*, 2008) have been excellently reviewed previously.

LPA generation in vascular diseases

Although the absolute concentration of circulating LPA varies considerably in different assays (Smyth *et al.*, 2008), it is clear that LPA is detectable in plasma in the low μM range. The generation of circulating LPA essentially requires the action of autotaxin (ATX), a secreted lysophospholipase D, which removes the polar head group from lysophospholipids (LPL), such as lysophosphatidylcholine (LPC) (Umezu-Goto *et al.*, 2002; Tokumura *et al.*, 2002c; Nakanaga *et al.*, 2010). Accordingly, plasma LPA levels of healthy subjects strongly correlate with the serum ATX activity (Hosogaya *et al.*, 2008) and are reduced by 50% in mice heterozygous for ATX (Tanaka *et al.*, 2006; van Meeteren *et al.*, 2006). Moreover, depletion of ATX completely prevents LPA production in serum (Tanaka *et al.*, 2006; Tsuda *et al.*, 2006), and ATX overexpression in transgenic mice increases LPA plasma levels (Pamuklar *et al.*, 2009). Inhibition studies demonstrated that continual ATX activity is required to maintain a steady state concentration of LPA in the circulation (Albers *et al.*, 2010; Gierse *et al.*, 2010; Gupte *et al.*, 2011). A boronic acid-based inhibitor in mice resulted in a rapid decline of LPA within minutes and the orally available ATX antagonist PF-8380 dose-dependently diminished the plasma LPA concentration by maximally 70% within 3 h (Albers *et al.*, 2010; Gierse *et al.*, 2010).

Adipocyte-derived ATX generates almost half of the LPA in plasma (Dusaulcy *et al.*, 2011). In obesity, insulin resistance in adipocytes has been linked to increased expression of ATX, implicating a potential role of LPA in the sequelae of the metabolic syndrome (Boucher *et al.*, 2005). Furthermore, hyperlipidaemia enhances the activity of circulating ATX in rabbits, which leads to enhanced generation of LPA from LPC in serum, and increases plasma LPA and adipose tissue expression of ATX in mice fed a high fat diet (Tokumura *et al.*, 2002a; Dusaulcy *et al.*, 2011).

Elevated circulating LPA levels may have pro-atherogenic effects, because systemic treatment with unsaturated LPAs has been shown to enhance atherosclerosis (Zhou *et al.*, 2011). In humans, plasma LPA levels correlate with LPC, ATX and LDL levels, indicating that hyperlipidaemia may contribute to the generation of LPA in the circulation (Dohi *et al.*, 2012). Moreover, circulating LPC is increased by hyperlipidaemia (Portman *et al.*, 1970; McConnell and Hoefner, 2006; Matsumoto *et al.*, 2007; Schmitz and Ruebsaamen, 2010). Plasma LPC, which is mainly bound to albumin is generated by endothelial lipase and by lecithin-cholesterol acyltransferase (LCAT) secreted from the liver (Wiesner *et al.*, 2009; Schmitz and Ruebsaamen, 2010). Prolonged (10–50 h) incubation of plasma at 37°C leads to a large increase in LPA from 0.5 to 15 μM that is due to LPC generation by LCAT and not by lipoprotein-associated (Lp-) PLA_2 activity (Aoki *et al.*, 2002).

Progression of atherosclerosis is associated with conditions of chronic inflammation and oxidant stress. Under these conditions, circulating LPC can be generated by group IIA secretory PLA_2 ($sPLA_2$), which is an acute-phase reactant, and by Lp-PLA_2 (identical to platelet-activating factor (PAF) acetylhydrolase) (Stafforini, 2009; Rosenson, 2010). $sPLA_2$ acts on lipoproteins and microvesicles (Fourcade *et al.*, 1995), whereas Lp-PLA_2 specifically catalyses the removal of the acyl group at the *sn-2* position of oxidatively truncated phospholipids present in oxidized LDL (ox-LDL) (Steinbrecher *et al.*, 1984). LDL particles oxidized to different degrees have been detected at increased levels in the circulation of patients with acute coronary syndromes (Holvoet *et al.*, 1998). Furthermore, electronegative LDL particles, which are pro-inflammatory by releasing chemokines from endothelial cells, are increased and physicochemically heterogeneous in familial hypercholesterolaemic patients (Sanchez-Quesada *et al.*, 2002; 2003). Moreover, circulating levels and enzymatic

activity of Lp-PLA_2 and $sPLA_2$ have been found to predict cardiovascular events (Packard *et al.*, 2000; Koenig and Khuseyinova, 2009; Thompson *et al.*, 2010). Lp-PLA_2 is primarily bound to electronegative LDL in the circulation, and the levels of Lp-PLA_2 positively correlate with the concentration of LDL (Packard *et al.*, 2000; Benitez *et al.*, 2003; Albert *et al.*, 2005). Of note, the Lp-PLA_2 inhibitor darapladib, which has currently been tested in two large cardiovascular outcome trials, limits atherosclerosis in mice (Charo and Taub, 2011; Wang *et al.*, 2011). The sPLA2 inhibitor varespladib reduces atherosclerosis in mice and a phase III cardiovascular outcome trial on the effects of varespladib is ongoing (Charo and Taub, 2011).

Although it remains to be determined whether pro-atherogenic effects of Lp-PLA_2 and $sPLA_2$ are related to increased LPA production, a very recent study showing a correlation between levels of Lp-PLA2, LPCs, LPA and pro-inflammatory cytokines in human plaques supports such a link (Goncalves *et al.*, 2012). Therefore, it can also be assumed that LPC levels elevated in hyperlipidaemia may lead to increased ATX-dependent LPA generation in the circulation. An interesting question in this regard is whether mice deficient in $sPLA_2$ or Lp-PLA_2 show decreased cellular LPA production or decreased LPA plasma levels. However, apart from one study that shows normal LPA generation of activated washed platelet suspensions prepared from mouse strains that are deficient in $sPLA_2$ expression (such as the C57BL/6 and C3H strains) (le Balle *et al.*, 1999), nothing is known about this topic.

LPA accumulates in human and mouse atherosclerotic lesions (Siess *et al.*, 1999; Bot *et al.*, 2010). LPA in atherosclerotic lesions is most likely to be derived from ATX-mediated hydrolysis of LPC. Although the expression of ATX in atherosclerotic lesions has not been studied, it has been shown that ATX can be secreted from arterial endothelial cells and that it is up-regulated in the arterial wall following vascular injury (Panchatcharam *et al.*, 2008; Zhou *et al.*, 2011). Structural analysis has suggested that ATX is capable of directing the LPA produced to the cognate GPCRs via binding of its somatomedin domains (SMD) to β3-integrins, thus contributing to localized LPA formation (Fulkerson *et al.*, 2011; Hausmann *et al.*, 2011; Nishimasu *et al.*, 2011). However, it remains to be determined whether local LPA production and delivery by ATX via binding to β3-integrins, which are highly expressed in various cell types of atherosclerotic lesions, contributes to LPA formation in the lesion (Hoshiga *et al.*, 1995).

In atherosclerotic lesions, the ATX substrate LPC is generated mainly by LDL oxidation. Polyunsaturated fatty acids in the *sn-2* position of phosphatidylcholine (PC) in the outer layer of the LDL particle undergo oxidative fragmentation to oxidized short-chain fatty acids (Siess, 2006). The oxidized PC molecules are then specifically hydrolyzed by Lp-PLA_2, which produces LPC (Steinbrecher *et al.*, 1984). LPC in the vessel wall accumulates during hyperlipidaemia, and LPC accumulation in a mouse atherosclerotic model precedes the progressive accumulation of LPA in atherosclerotic tissue (Portman *et al.*, 1970; Bot *et al.*, 2010).

In addition, LPA in atherosclerotic lesions might derive indirectly from microparticles or microvesicles that are shed from activated and apoptotic cells (e.g. macrophages and VSMCs) and accumulate in inflamed atherosclerotic lesions. Membrane phospholipids of microvesicles, but not intact cells have been reported to be accessible to hydrolysis by $sPLA_2$, and the concentration of lysophospholipids was found to be increased in microvesicles isolated from inflammatory fluids (Fourcade *et al.*, 1995). Thus, this enzyme could also generate lysophospholipids from microparticles for ATX-dependent LPA formation in inflamed atherosclerotic lesions, although the role of microparticles in the production of lesional LPA remains to be determined.

Previous reports have described increased formation of LPA, preferentially of the alkyl-LPA species, during mild oxidation of LDL (Siess *et al.*, 1999; Zhang *et al.*, 2004), suggesting that a pathway of LPA formation during LDL oxidation may exist, which may be ATX-independent. However, the possibility that ATX, similar to other plasma proteins, might also be associated with LDL (Hoofnagle and Heinecke, 2009) cannot be excluded, although direct evidence is lacking.

Platelet activation importantly contributes to LPA formation in blood. Platelet depletion reduces the serum LPA levels by 50% in rats (Aoki *et al.*, 2002). During blood clotting, the LPA concentration rises from low micromolar plasma concentrations (0.1–1 μM) up to 10 μM in serum predominantly by an increase of acyl-LPA species containing polyunsaturated fatty acids (mainly 18:2 and 20:4 fatty acids) (Siess, 2002; Tigyi, 2010). Activation of isolated platelets induces the formation of intracellular LPA via a pathway that involves the PLC-catalysed hydrolysis of phosphoinositides leading to the formation of diacylglycerol that is rapidly phosphorylated by diglyceride kinase to phosphatidic acid (PA) (Siess, 2002). Subsequently, degradation by platelet PLA_2 and PLA_1 enzymes specific for PA leads to the formation of 1-acyl-LPA and 2-acyl-LPA, respectively (Billah *et al.*, 1981; Gaits *et al.*, 1997). Intracellularly produced LPA is not released into the extracellular medium (Watson *et al.*, 1985). LPA in the medium of activated platelets can only be detected in the presence of albumin (Eichholtz *et al.*, 1993). Recent studies indicate that the small amounts of extracellular LPA that can be detected upon activation of isolated platelets are due to the activity of platelet-bound ATX (Pamuklar *et al.*, 2009; Fulkerson *et al.*, 2011). Thus, it seems likely that extracellular LPA detected upon activation of washed platelets is formed by platelet-bound ATX from LPC, and albumin by binding platelet-released LPC plays an essential role in this process.

In contrast to activation of platelets in buffer, platelet activation in the presence of plasma leads to a large increase in extracellular LPA. This occurs by a multistep process involving the generation of LPL via intracellular PLAs and secreted PLA_1 and/or PLA_2 enzymes (Aoki *et al.*, 2002; Sano *et al.*, 2002). Very recent studies demonstrating the binding of ATX to β3-integrins of activated platelets (Fulkerson *et al.*, 2011) and the identification of a new PLA enzyme secreted from platelets (Bolen *et al.*, 2011) unravel how platelet activation may lead to the large increase in certain molecular LPA species (18:2-acyl-LPA and 20:4 acyl-LPA) in serum. The platelet PLA enzyme that may feed plasma ATX with LPLs upon platelet activation is acyl-protein thioesterase 1 (APT1), also known as lysophospholipase A-I (LYPLA-I) (Bolen *et al.*, 2011). APT1 is released from activated platelets, acts like PLA_1 and generates a pool of *sn*-2-esterified lysophospholipids containing mainly C18:2 and C20:4 (Bolen *et al.*, 2011). The thermodynamically unstable *sn*-2-esterified

lysophospholipids undergo acyl migration resulting in *sn-1*-esterified lysophospholipids, which are the preferred substrates of ATX (Bolen *et al.*, 2011). Subsequently, ATX bound to activated β3-integrins on the activated platelet membrane via its two SMD produces LPA species containing mainly 18:2 and 20:4 fatty acids (Bolen *et al.*, 2011; Fulkerson *et al.*, 2011). This platelet-dependent LPA production may be crucial for vascular repair, for example, after erosion of atherosclerotic plaques or after stent implantation, where activated platelets rapidly adhere to the denuded vascular surface.

Extracellular LPA is degraded by the ecto-activities of lipid phosphate phosphatases (LPPs) (Morris *et al.*, 2009; Samadi *et al.*, 2011). LPPs hydrolyze a large variety of bioactive lipid phosphates and pyrophosphates; their active site resides on the outer surface of plasma membranes. Three different mammalian LPP isoforms are known. Whereas i.v. injected LPA has a half-life of only 3 min in mice, an extended half-life of circulating LPA up to 12 min and increased levels of plasma LPA have been found in LPP1-deficient mice (Tomsig *et al.*, 2009). It is therefore likely that a balance between ATX and LPP activities controls the concentration of extracellular LPA and thus LPA receptor activation in a dynamic manner (Samadi *et al.*, 2011).

In addition to LPA, cyclic phosphatidic acid (1-acyl-sn-glycerol-2,3-cyclic phosphate; cPA) probably formed by ATX, has been identified in human serum (Kobayashi *et al.*, 1999; Tsuda *et al.*, 2006; Fujiwara, 2008; Shan *et al.*, 2008b). cPA is an LPA analogue in which the *sn-2* hydroxyl group has formed a ring structure with the *sn-3* phosphate. Although the function of extracellular cPA on vascular cells and blood cells is largely unknown (Fujiwara, 2008; Tigyi, 2010), intracellular cPA, formed by PLD activation, functions as inhibitor of PPARγ and might thereby modify atherosclerotic processes (Tsukahara *et al.*, 2010).

Measurement of plasma LPA and circulating LPA levels in cardio- and cerebrovascular clinical studies

The two primary methods that are used to measure LPA in plasma are liquid chromatography-electrospray ionization tandem MS (LC-ESI-tandem MS) and enzymatic assays. Two enzymatic methods have been described: (i) a radioenzymatic assay uses recombinant LPA acid acyltransferase and a radioactive fatty acid to measure the formation of radiolabeled PA (Saulnier-Blache *et al.*, 2000); and (b) an enzymatic cycling assay uses lysophospholipase, which generates glycerol 3-phosphate, followed by enzymatic cycling using glycerol 3-phosphate oxidase and glycerol 3-phosphate dehydrogenase. The amplified concentrations of hydrogen peroxide are then measured colorimetrically (Kishimoto *et al.*, 2003). Both of these methods yielded LPA plasma levels (0.07–0.1 μM) that were 10-fold lower than the levels measured by the LC/MS/MS method (0.7–1 μM) (Baker *et al.*, 2001; Liebisch and Scherer, 2012).

The determination of plasma LPA is not trivial, and the conditions of sample preparation are important for accurate measurement. The majority of LPA in the circulation is bound to albumin (Tigyi *et al.*, 1991; Tigyi and Miledi, 1992), and acidic extraction methods are required. The addition of strong acids may lead to conversion of plasma LPC to LPA in plasma samples, leading to an artificial increase in LPA (Shan *et al.*, 2008a; Liebisch and Scherer, 2012). This is important because plasma LPC levels are approximately 100–1000 times higher than that of LPA. To circumvent this problem, the butanolic extraction procedure (pH 4) described by Baker *et al.* is often the method of choice for LPA extraction (Baker *et al.*, 2001; Liebisch and Scherer, 2012). Furthermore, direct mass spectrometric analysis of crude lipid extracts should be avoided when determining the levels of plasma LPA. In-source fragmentation of LPC and lysophosphatidylserine (LPS) to LPA has been reported after direct flow injection during plasma LPA analysis (Zhao and Xu, 2009; Liebisch and Scherer, 2012). LPC can lose its choline group at the ion source before the parent ions are detected, giving rise to signals that are indistinguishable from endogenous LPA.

Activated platelets also contribute significantly to LPA generation in the blood, which is an additional factor that makes it difficult to accurately determine the circulating LPA levels. Platelets are activated easily after venipuncture, and it is impossible to immediately inhibit platelet activation before the blood comes into contact with the anticoagulant and additional platelet inhibitors that are perhaps present in the blood collection cuvette. Similar problems have previously been encountered when determining the concentration of circulating thromboxane and other substances that are released from activated platelets (FitzGerald *et al.*, 1987). However, platelet activation during the subsequent handling of the blood such as during the centrifugation of blood to obtain plasma can be controlled. The lowest plasma concentrations of LPA (approximately 0.1 μM, measured by the enzymatic cycling assay) (Kishimoto *et al.*, 2003) and LPC (approximately 190 μM) were found when blood was drawn into 7.5 mM EDTA plus a mixture of 10% (v v^{-1}) citrate, theophylline, adenosine and dipyridamole (Nakamura *et al.*, 2007). The latter three substances are platelet inhibitors. In blood samples that were collected in this manner, the LPA concentration was significantly higher in women (0.1 μM) than in men (0.077 μM), and a positive correlation between the plasma LPA concentration and serum lysophospholipase D (lysoPLD) activity was found. However, the LPA concentration could be correlated with the plasma LPC concentration only in men (Hosogaya *et al.*, 2008).

In most of the clinical studies that report LPA plasma levels, no precautions have been taken to control for the artificial contribution of LPA production from activated platelets *in vitro* during blood handling and processing. For future studies, the inclusion of a pharmacological ATX inhibitor would be ideal to circumvent this problem. After i.v. injection of a boronic acid-based inhibitor of ATX into mice, a rapid decrease in the total plasma LPA concentration was observed; this finding was interpreted to indicate that there is a dynamic turnover of LPA in the circulation (Albers *et al.*, 2010). Another explanation for these results could be that the injected ATX inhibitor attenuated the *in vitro* formation of LPA during blood handling.

In a recent cross-sectional study of consecutive patients, it was reported that patients with acute coronary syndrome have significantly increased plasma LPA levels (0.54 μM) compared with patients with stable angina pectoris (0.36 μM)

or angiographically normal coronary arteries (0.41 μM) (Dohi *et al.*, 2012). In this study, LPA was measured enzymatically as previously described (Kishimoto *et al.*, 2003); however, special precautions to inhibit LPA formation during blood handling *in vitro* (Nakamura *et al.*, 2007) were not taken. The blood was collected into EDTA-containing tubes (Dohi *et al.*, 2012), and the LPA plasma levels of the patients with angiographically normal coronary arteries (approximately 0.4 μM) were approximately four times higher than the LPA plasma levels that have been reported previously in healthy people. The interpretation of this clinical study is therefore difficult as patients with unstable angina pectoris might exhibit higher LPA plasma levels because their platelets are more easily activated *in vitro*.

Another clinical study investigated the influence of acetylsalicylate on plasma LPA levels in patients with ischaemic cerebral vascular diseases (Li *et al.*, 2008). Elevated LPA levels were found in patients with ischaemic cerebrovascular disease (3.11 μM) compared with healthy controls (1.77 μM). Daily administration of aspirin (100 mg) for 1 month significantly lowered the LPA levels in the patients from 4.06 to 2.41 μM. The authors concluded that their findings support a close association between increased plasma LPA levels and platelet activation. Again, it is difficult to interpret whether the effect of aspirin on the plasma LPA levels occurred *in vivo* or *in vitro*.

A separate clinical study investigated, whether there is a relationship between LPA levels and the prevalence of silent brain infarction (SBI) in patients with non-valvular atrial fibrillation (NVAF) (Li *et al.*, 2010). The plasma LPA levels in the NVAF patients with SBI were significantly higher than those in the control patients ($P < 0.01$) or the NVAF patients without SBI. The authors suggested that LPA might be a novel marker for estimating the status of platelet activation and the risk for SBI onset in NVAF patients.

The question when interpreting all of these studies is whether the plasma LPA that is measured truly reflects the circulating LPA concentration or whether it was formed *in vitro* from platelets that were activated during blood handling. In future studies, the addition of a pharmacological ATX inhibitor to the blood collection tube is recommended to minimize LPA formation during blood handling. Moreover, the measurement of total plasma LPA levels may not be sufficient to predict the cardiovascular risk, because the thrombogenicity and atherogenicity of LPA crucially depends on the type of bondage of the fatty acyl chain to the glycerol backbone (ester or ether) and the saturation of the fatty acid; alkyl-LPA species are more potent platelet activators than the corresponding acyl-LPA species (Simon *et al.*, 1982; Tokumura *et al.*, 2002d; Rother *et al.*, 2003), and only unsaturated acyl-LPA species are atherogenic (Yoshida *et al.*, 2003; Zhang *et al.*, 2004; Zhou *et al.*, 2011). In line with these findings, elevated levels of LPA during pregnancy, which is not associated with increased cardiovascular risk, is due to a rise predominantly of saturated LPA, such as LPA16:0 (Tokumura *et al.*, 2002b). Furthermore, increased circulating LPA has been found in patients with chronic hepatitis C and liver fibrosis and in experimentally induced liver fibrosis (Watanabe *et al.*, 2007a,b). In contrast to hepatitis C infection, which is associated with increased cardiovascular risk, liver cirrhosis does not lead to accelerated atherosclerosis (Petta *et al.*, 2011; Purnak *et al.*, 2011; Adinolfi *et al.*, 2012). Unfortunately, it is currently unknown which LPA species are elevated in the different forms of liver diseases.

Further studies on the circulating levels of the various LPA species may help to clarify the role of elevated circulating LPA levels in atherogenesis. These could also give a hint for the origin of LPA, because circulating LPA might derive from activated platelets (which produce mainly 18:2-acyl-LPA and 20:4 acyl-LPA), microparticles originating from damaged or apoptotic cells (their LPA species are unknown) or other cell types (adipocytes). However, in general, the relevance of dosing LPA in plasma could be questioned, because LPA is mainly generated locally in the circulation and acts locally.

Effects of LPA on blood and vascular cells

LPA has multiple effects on blood cells and cells of the vessel wall (Siess, 2002; Smyth *et al.*, 2008). In platelets, it induces directly shape change, and it stimulates platelet aggregation and secretion only in synergy with other platelet stimuli (Rother *et al.*, 2003; Haseruck *et al.*, 2004). In human monocytes, LPA increases cytosolic Ca^{2+} (Fueller *et al.*, 2003), and in macrophages, it stimulates cell survival and ox-LDL uptake (Koh *et al.*, 1998; McIntyre *et al.*, 2003). In endothelial cells, LPA stimulates cell migration (Panetti *et al.*, 2000; 2004; Ptaszynska *et al.*, 2010), chemokine secretion (Lin *et al.*, 2006; Zhou *et al.*, 2011), adhesion molecule expression (Rizza *et al.*, 1999; Zhou *et al.*, 2011), actin stress fibre formation and cell contraction (Siess *et al.*, 1999; Hirakawa *et al.*, 2004). In confluent endothelial cells *in vitro* or in endothelium *in vivo*, LPA can either increase (van Nieuw Amerongen *et al.*, 2000; Hirase *et al.*, 2001; Sarker *et al.*, 2010) or decrease (Alexander *et al.*, 1998; Minnear *et al.*, 2001) endothelial permeability. LPA also co-operates with VEGF to stimulate angiogenesis (Tanaka *et al.*, 2006; van Meeteren *et al.*, 2006; Ptaszynska *et al.*, 2010). In VSMCs, LPA stimulates cell contraction, leading to increased vascular tone, cell migration, and proliferation [for ref. see (Siess, 2002; Smyth *et al.*, 2008)].

The specific cellular effects of LPA depend on the species of the LPA molecule, and the origin and the vascular environment of the target cells. There are multiple molecular species of LPA that have been described in biological fluids and that are present in plasma and the lipid-rich core of atherosclerotic plaques (Baker *et al.*, 2001; Rother *et al.*, 2003). The fatty acid is mainly attached at the *sn-1* position of the glycerol backbone via an ester (1-acyl-LPA) or ether bond (1-alkyl-LPA, 1-alkenyl LPA). The fatty acids, which are of predominantly 16-, 18- and 20-carbon lengths, can be saturated, mono- or poly-unsaturated. Additionally, 2-acyl regioisomers of LPA have been detected; however, *sn-2*-esterified lysophospholipids are unstable and undergo acyl migration to yield *sn-1*-esterified lysophospholipids (Bolen *et al.*, 2011).

The types of LPA molecules that are present determine, qualitatively or quantitatively, the biological response of the cell. For example, acyl-LPA that contain unsaturated but not saturated fatty acid causes the phenotypic dedifferentiation of cultured VSMCs and elicits neointima formation in a non-injury model of the rat carotid artery (Hayashi *et al.*, 2001;

Yoshida *et al.*, 2003; Zhang *et al.*, 2004; Subramanian *et al.*, 2010). Furthermore, LPA (20:4), but not LPA (18:0), triggers the adhesion of monocytes to the vessel wall and enhances the progression of atherosclerosis (Zhou *et al.*, 2011). In platelets, alkyl-LPA (16:0) and acyl-LPA (20:4) are approximately 20- and 7-fold more potent than acyl-LPA (16:0), respectively (Tokumura *et al.*, 2002d; Rother *et al.*, 2003; Haseruck *et al.*, 2004). In cells that express individual recombinant LPA receptors, most of the receptors show an agonist preference for mono- or poly-unsaturated acyl-LPA over saturated acyl-LPA (Tigyi, 2010). However, whether endogenous LPA receptors, which are expressed at much lower levels in mammalian cells, behave similarly is not known.

The type of LPA response can also vary between species. For example, human and cat platelets, but not rodent (rat and mouse) platelets, are activated by LPA (Schumacher *et al.*, 1979; Tokumura *et al.*, 1981). Interestingly, mouse platelets are inhibited by LPA (Pamuklar *et al.*, 2009). Additionally, the type of vascular bed is important in considering the effect of LPA on endothelial cells because the endothelium shows a pronounced heterogeneity. There are varied biomechanical and biochemical inputs along the vasculature that alter the properties of the endothelium, and therefore its response to the same pathophysiological stimuli will be different (Aird, 2005). Thus, *in vivo* studies on the action of LPA on endothelial cells will be particularly important as these allow the study of endothelial cells in healthy and atherosclerotic arterial vessels *in situ* (Zhou *et al.*, 2011). In contrast, *in vitro* culture of endothelial cells cannot preserve their vascular bed-specific phenotype, and cell culture also leads to a loss of glycocalyx on the cell surface, which is an important endothelial interface with circulating blood (Chappell *et al.*, 2009).

An important consideration when investigating the activity of circulating LPA is that plasma albumin might limit the activity of LPA. Albumin binds LPA with a stoichiometry of 3 mol of LPA to 1 mol of albumin (Tigyi and Miledi, 1992; Thumser *et al.*, 1994), and albumin dose-dependently inhibits LPA-induced platelet shape change and aggregation (Tokumura *et al.*, 2002d; Haseruck *et al.*, 2004; Khandoga *et al.*, 2008). This finding explains the observation that approximately 1000-fold higher LPA concentrations (0.5–20 μM) are required to stimulate platelets in plasma and blood, as compared with the stimulation of washed platelet suspensions. It is therefore unlikely that the low LPA concentrations found in plasma (total LPA: 0.1–1 μM) affect circulating platelets, and these low levels may also not affect other blood cells or endothelial cells as well.

For many of the effects of LPA on blood cells and cells of the vessel wall, the LPA receptors that are responsible have not been identified. The availability of mice that are deficient for individual LPA receptors (Choi *et al.*, 2010) has advanced our knowledge of the involvement of specific LPA receptors in atherosclerotic processes (Panchatcharam *et al.*, 2008). Often, *in vitro* studies of LPA receptor expression in individual cells have to rely on quantitative PCR analysis of mRNA transcripts because the possibly low endogenous expression of LPA receptor proteins is difficult to determine by immunoblot with specific LPA-receptor antibodies. Furthermore, the fact that one cell type often expresses many different LPA receptors, and that a single LPA receptor can couple to different G-proteins in the same cell (Choi *et al.*, 2010), thereby stimulating a complex LPA signalling network, makes it difficult to dissect the function of individual LPA receptors. However, studies using the siRNA technology to down-regulate individual LPA receptors in vascular cells, blood cells or isolated arteries are helpful to assign a specific LPA receptor to its cellular or vascular effect (Subramanian *et al.*, 2010; Khandoga *et al.*, 2011; Zhou *et al.*, 2011).

LPA in acute atherothrombosis

Myocardial infarction and ischaemic stroke are leading causes of morbidity and mortality in humans. The trigger for approximately 70% of myocardial infarctions is plaque rupture, and the erosion of vulnerable atherosclerotic plaques accounts for the other 30%. These events lead to the exposure of thrombogenic plaque material to circulating blood (Fernandez-Ortiz *et al.*, 1994; van Zanten *et al.*, 1994; Toschi *et al.*, 1997; Kolodgie *et al.*, 2004). Subsequent platelet activation and fibrin formation can lead to the development of an occluding thrombus with possibly fatal consequences for the patient. LPA may play different roles in thrombus formation after erosion and plaque rupture. After plaque erosion, the main thrombogenic stimulus is possibly subendothelial versican-hyaluronan matrix (Kolodgie *et al.*, 2004), whereas after plaque rupture, collagen of the disrupted cap and possibly LPA of the plaque core are the platelet stimuli (van Zanten *et al.*, 1994; Siess *et al.*, 1999; Corti and Badimon, 2002; Rother *et al.*, 2003; Penz *et al.*, 2005; Nakanaga *et al.*, 2010). The lipid core contains alkyl-LPA and acyl-LPA species with high platelet-activating potency (Rother *et al.*, 2003) (Figure 1). On the other hand, LPA locally formed by ATX bound to activated platelets that cover eroded plaques might, in concert with other platelet-derived mediators such as sphingosine 1-phosphate (S1P) and VEGF, activate neighbouring endothelial cells to migrate and proliferate, and thereby help in healing the endothelial defect (Panetti *et al.*, 2000; 2004; Ptaszynska *et al.*, 2010).

LPA that is produced by activated platelets after plaque erosion or rupture may play a role as a positive feedback mediator of platelet activation. However, in a previous study, no inhibitory effects of LPA receptor antagonists were found on aggregation of washed platelets stimulated by collagen or thrombin, indicating that the small amounts of LPA that were generated by stimulated, washed platelets do not mediate or support stimulus-induced platelet aggregation (Haseruck *et al.*, 2004). Whether LPA plays a role as a positive feedback mediator of platelet activation in stimulated blood where ATX-dependent LPA formation rises drastically is not known.

Thrombin activation of isolated platelets leads to the activation of the integrin αIIbβ3 and binding of extracellular ATX to the β3 integrin through its tandem somatomedin B (SMB) domains (Fulkerson *et al.*, 2011; Hausmann *et al.*, 2011). Echistatin, an arginine–glycine–aspartic acid (RGD)-containing peptide, and a monoclonal antibody against integrin αIIbβ3 (10E5) reduced platelet adhesion to ATX and inhibited ATX-mediated LPA formation by thrombin-activated, isolated platelets (Fulkerson *et al.*, 2011). However, the RGD sequence of the tandem SMB domains is not involved in the binding of ATX to the β3 integrin of activated

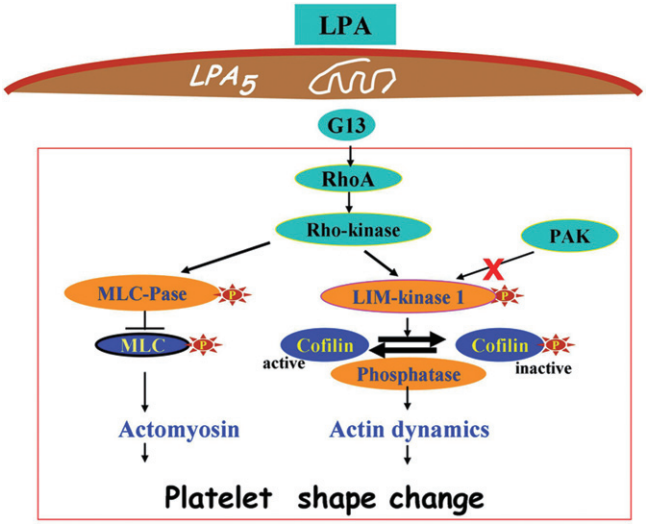

Figure 1

LPA-induced platelet signalling during platelet shape change. Activation of the LPA_5 receptor coupled to the heterotrimeric G13 protein stimulates Rho and Rho-kinase. The subsequent bifurcating pathway directed to either the myosin-binding subunit of MLC phosphatase or the LIM-kinase 1 leads to enhanced phosphorylation of MLC and stimulation of phospho-cofilin turnover, respectively. Phosphorylated myosin develops actin-activated ATPase activity, interacts with F-actin, and assembles into filaments, whereas cofilin regulates actin dynamics by enhancing both actin-polymerization and actin filament severing. These cytoskeleton changes underlie the folding of the surface membrane, the formation of pseudopods and the contractile wave centralizing the secretory granules during platelet shape change.

platelets, as demonstrated by an ATX–RGE mutant that showed a similar binding to activated platelets as that of wild-type ATX (Hausmann *et al.*, 2011). Although it has not been tested directly, it is likely that LPA formation in blood also depends on binding of ATX to the β3 integrins of activated platelets. In blood, ATX, which is present at a concentration of approximately 100 nM in plasma (Nakamura *et al.*, 2008), has to compete with the much higher plasma concentration of fibrinogen (5–8 μM) for binding to the β3 integrin on activated platelets.

Healthy people display individual heterogeneity of the LPA-induced platelet aggregation response when measured in washed platelets, PRP or blood (Haseruck *et al.*, 2004; Pamuklar *et al.*, 2008). However, all of the blood donors showed a similar shape change response when tested in blood (Haseruck *et al.*, 2004). LPA-induced platelet aggregation was completely dependent on ADP-mediated activation of $P2Y_1$ and $P2Y_{12}$ receptors in whole blood (Haseruck *et al.*, 2004). Thus, LPA does not directly induce human platelet aggregation in blood, as LPA-induced platelet aggregation requires the presence of extracellular ADP. In stirred blood, ADP may be either secreted from platelet-dense granules or released from red cells. In a recent study using isolated platelets, the individual heterogeneity of LPA-induced platelet aggregation was hypothesized to be due to LPA-induced activation of an inhibitory pathway in the non-responders (Pamuklar *et al.*, 2008).

Low nM concentrations of LPA directly induce shape change in washed platelets (Siess *et al.*, 1999; Rother *et al.*, 2003). LPA binds to GPCRs on the platelet surface, and the signal that is emitted by the activated platelet receptor is transduced by the heterotrimeric G_{13} protein to activate Rho and Rho-kinase (Bauer *et al.*, 1999; Klages *et al.*, 1999; Gratacap *et al.*, 2001; Moers *et al.*, 2003). Rho-kinase phosphorylates the 130-kD myosin-binding subunit of myosin phosphatase, thereby decreasing the activity of myosin phosphatase and increasing myosin light chain (MLC) phosphorylation (Kimura *et al.*, 1996; Bauer *et al.*, 1999; Retzer and Essler, 2000). Rho-kinase also phosphorylates and activates LIM-kinase 1, which increases the turnover of phospho-cofilin (Pandey *et al.*, 2006; 2007). These biochemical pathways converge in the remodelling of actin–myosin structures that underlie platelet shape change (Figure 1). Cytosolic Ca^{2+} increase, Rac activation and p21-activated kinase (PAK) are not involved in LPA-induced shape change (Maschberger *et al.*, 2000; Pandey *et al.*, 2007). High concentrations of LPA (10 μM) induce an increase in cytosolic Ca^{2+} in washed platelets that is primarily due to the stimulation of Ca^{2+} entry. This leads to cofilin dephosphorylation and secretion; the latter response requires, in addition, integrin αIIβ3 outside-in signalling (Maschberger *et al.*, 2000; Pandey *et al.*, 2007). LPA does not activate the heterotrimeric G-protein G_i in platelets, yet it shows a strong synergism in the induction of platelet aggregation with platelet stimuli such as adrenaline and ADP that activate G_i (Rother *et al.*, 2003; Haseruck *et al.*, 2004).

Until recently, the identity of the LPA receptor that mediates platelet shape change remained obscure. Human platelets express mRNA for the Edg receptors LPA_{1-3}, and the purinergic cluster LPA_4 (GPR23), LPA_5 (GPR92), LPA_6 ($P2Y_5$), and the putative receptors GPR87 and $P2Y_{10}$ (Amisten *et al.*, 2008; Khandoga *et al.*, 2008; Pamuklar *et al.*, 2008). The receptors that are most abundantly expressed at the mRNA level are LPA_4 and LPA_5 (Amisten *et al.*, 2008; Khandoga *et al.*, 2008). A previous study suggested that LPA_1 and LPA_3 play a role in inducing platelet shape change based on the ability of the LPA receptor subtype-specific antagonist diacylglycerol pyrophosphate to inhibit LPA-induced platelet shape change. However, the inhibitory effect of this compound increased with the time of pre-incubation (Rother *et al.*, 2003), which is not typical for a receptor antagonist. Two recent studies described the involvement of LPA_4 and LPA_5 in mediating LPA-induced platelet shape change (Khandoga *et al.*, 2008; Williams *et al.*, 2009). However, firm evidence that these receptors were the functional platelet LPA receptors was lacking. The LPA response of platelets did not match the pharmacological properties of the LPA_4 and LPA_5 receptors in heterologous expression systems (Khandoga *et al.*, 2008), and the pharmacological receptor agonists and antagonists that were used were not selective for LPA_5 (Williams *et al.*, 2009).

More recently, it was demonstrated that LPA-induced shape change in two human megakaryocytic cell lines was inhibited by siRNA against LPA_5, but not by knock-down of each of the other receptors $LPA_{1-4,6,7}$ (Khandoga *et al.*, 2011). The rank order of activation by LPA species in these cells, with alkyl-LPA 18:1 and alkyl-LPA 16:0 being the most potent, was similar to that of human platelets, supporting the hypothesis that LPA_5 is the functional LPA receptor-mediating platelet shape change (Figure 1). Importantly, siRNA against LPA_5 also

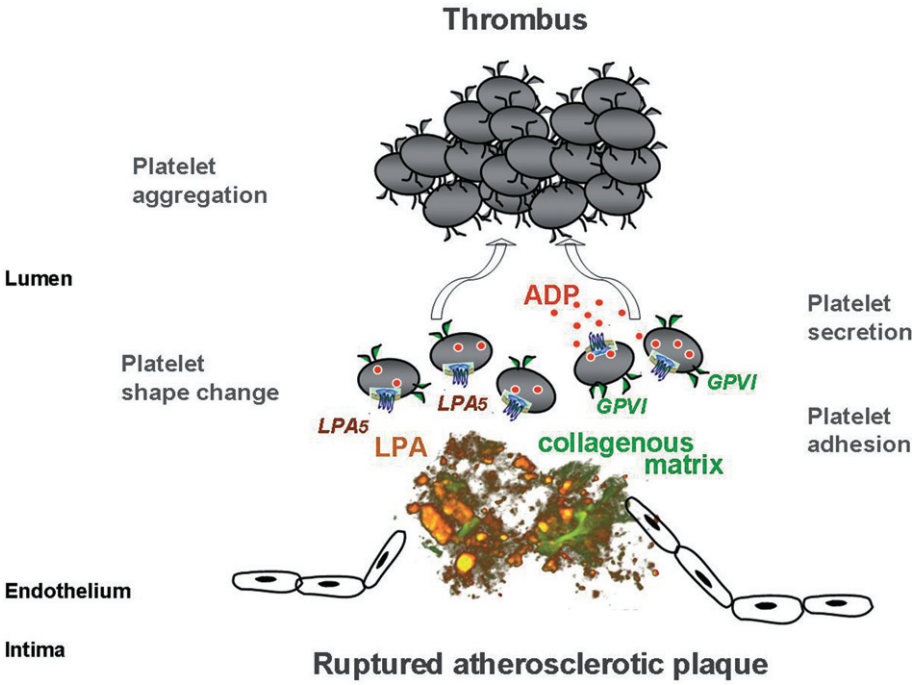

Figure 2

Hypothetical role of plaque LPA and the platelet LPA_5 receptor in acute atherothrombosis after plaque rupture. LPA in the lipid core of atherosclerotic plaques may act as a cofactor with platelet-adhesive matrix proteins such as collagen type I and III in platelet activation (van Zanten *et al.*, 1994; Penz *et al.*, 2005; Schulz *et al.*, 2008). These matrix proteins are over-expressed in plaques as compared with healthy arterial intima. LPA induces, through binding to the LPA_5 receptor, shape change of circulating platelets and has a synergistic effect with ADP at inducing platelet aggregation and thrombus formation. ADP is secreted from dense granules of platelets adhering to the collagenous matrix of the ruptured cap. GPVI, glycoprotein VI.

inhibited shape change of the megakaryocytic cells that was induced by the lipid-rich core of human atherosclerotic plaques (Khandoga *et al.*, 2011). Thus, LPA_5 may be a novel target for anti-thrombotic therapy for patients with ischaemic cardio- and cerebrovascular disease.

Upon plaque rupture, LPA exposed by the lipid core might, by binding to LPA_5, induce platelet shape change and stimulate, in synergy with ADP, platelet aggregation and thrombus formation (Haseruck *et al.*, 2004; Rother *et al.*, 2003) (Figure 2). ADP can derive from damaged red cells (Born and Wehmeier, 1979) and from activated platelets adhering to the collagenous matrix of the ruptured cap and secreting their granule contents (Penz *et al.*, 2005; Reininger *et al.*, 2010) (Figure 2). Thus, plaque LPA may act as a cofactor in acute atherothrombosis. However, the importance of LPA in the lipid-rich core for platelet activation in whole blood remains to be demonstrated. Specific LPA_5 receptor antagonists will be helpful to answer this question. Indirect evidence supports a role for plaque LPA in plaque-induced platelet activation and thrombus formation. Low (sub-μM) concentrations of alkyl-LPA species, that may be reached locally after plaque rupture, activate platelets in blood (Haseruck *et al.*, 2004), and aspirin is equally ineffective at inhibiting LPA-triggered platelet aggregation in blood (Haseruck *et al.*, 2004) and plaque-induced platelet thrombus formation under arterial flow conditions (Penz *et al.*, 2007).

LPA in atherosclerosis

Hypercholesterolaemia promotes the continuous recruitment of circulating monocytes to the arterial wall, which drives the progression of atherosclerotic plaques (Swirski, 2006). LDL enters the vessel wall and is retained in the subendothelial space, where LDL is oxidatively modified. However, the type and degree of LDL modifications are diverse, resulting in distinct biological activities. Oxidized LDL is generally divided into two main categories: minimally modified or mildly oxidized LDL (MM–LDL or mox-LDL) and extensively oxidized LDL (ox-LDL) (Stocker and Keaney, 2004; Levitan *et al.*, 2010). Although both are chemically modified, MM–LDL differs from oxLDL, because it still binds to the LDL receptor and is not recognized by most scavenger receptors (Stocker and Keaney, 2004; Levitan *et al.*, 2010). In contrast to native LDL, MM-LDL can induce the adhesion of monocytes to endothelial cells by up-regulating the CXC chemokine, CXCL1, on the endothelial surface (Berliner *et al.*, 1990; Schwartz *et al.*, 1994). Oxidized LDL or components of LDL that are released during the oxidation, like oxidized phospholipids, trigger the inflammatory response by activating endothelial cells (Hansson and Hermansson, 2011; Weber and Noels, 2011). Activated endothelial cells express adhesion molecules and chemokines that direct the adhesion and transmigration of circulating monocytes. These monocytes

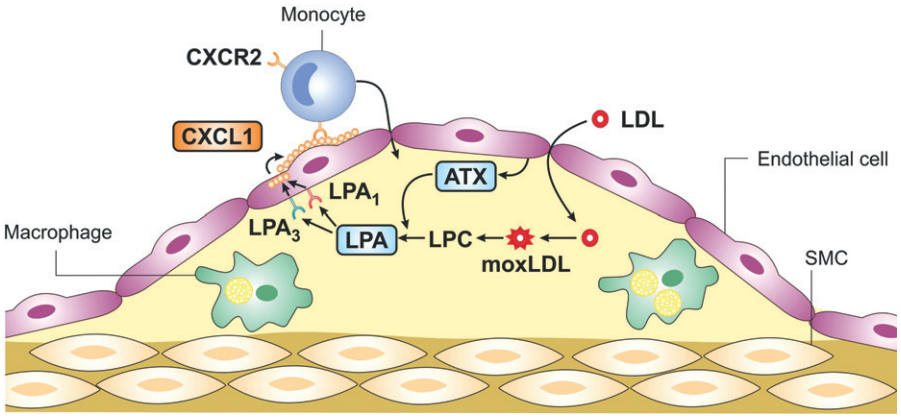

Figure 3

LPA promotes the accumulation of macrophages in atherosclerotic lesions. The moxLDL leads to increased formation of LPC, which is converted by endothelial-derived ATX into LPA. LPA triggers the release of the chemokine CXCL1 from endothelial cells through the activation of LPA_1 and LPA_3. CXCL1 is immobilized on the endothelial surface and induces the adhesion of monocytes to the vessel wall via its receptor CXCR2 on monocytes. These monocytes migrate into the subendothelial space and transform into macrophages, which are the primary cells in early atherosclerotic plaques.

transform into macrophages in the vessel wall, where they engulf ox-LDL and propagate the inflammatory reaction in concert with T-cells. During the progression of atherosclerosis, SMCs accumulate and form a fibrous cap that encloses a highly thrombogenic core comprising extracellular lipids.

LPA accumulates during atherogenesis induced by perivascular collar placement in $Apoe^{-/-}$ mice and is increased in the lipid core region of human atherosclerotic plaques (Rother *et al.*, 2003; Bot *et al.*, 2010). *In vitro*, LPA has been shown to activate NF-κB and increase the adhesion of monocytes to endothelial cells under static conditions by up-regulating adhesion molecules and chemokines, such as intracellular adhesion molecule-1 (ICAM-1), E-selectin, and vascular cell adhesion molecule-1 (VCAM-1) (Palmetshofer *et al.*, 1999; Rizza *et al.*, 1999; Lee *et al.*, 2004; Lin *et al.*, 2007). Whereas LPA-induced endothelial ICAM-1 expression is mediated by the LPA_1 receptor, both LPA_1 and LPA_3 are involved in the chemotactic activity generated by the secretion of IL-8 and chemokine ligand 2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1) from LPA-stimulated endothelial cells (Lee *et al.*, 2004; Gustin *et al.*, 2008b). Additionally, the endothelial release of pentraxin-3 is induced by LPA and enhances monocyte migration (Gustin *et al.*, 2008a). However, the role of LPA-induced endothelial pentraxin-3 secretion in atherogenesis is unclear.

In murine carotid arteries, atherogenic monocyte adhesion under flow conditions is primarily mediated by the CXC chemokine CXCL1 (human GRO-α/murine KC), which is in contrast to CCL2 immobilized on the endothelial surface (Weber *et al.*, 1999; Huo *et al.*, 2001). Whereas most chemokines are transcriptionally up-regulated, CXCL1 is stored in intracellular vesicles of endothelial cells (Oynebraten, 2004; Zhou *et al.*, 2011). The modified LDL-induced secretion of endothelial CXCL1 is mediated by an unsaturated LPA species and requires the activity of ATX (Zhou *et al.*, 2011). This secretagogue effect of LPA is mediated by the $LPA_{1/3}$-induced activation of Rho-associated coiled-coil containing protein kinase. However, activation of NF-κB by unsaturated LPA induces transcriptional up-regulation of CXCL1 in endothelial cells, suggesting a biphasic response (Zhou *et al.*, 2011). This mechanism of LPA on CXCL1 is crucial in promoting atherogenic monocyte recruitment and atherosclerosis *in vivo* (Figure 3). Hyperlipidaemia-induced monocyte adhesion to carotid arteries is almost completely abolished by pharmacological inhibition of $LPA_{1/3}$, indicating that LPA is an important mediator of the pro-atherogenic effects of ox-LDL (Zhou *et al.*, 2011). This reduced monocyte recruitment may explain the inhibition of atherogenesis observed by blocking $LPA_{1/3}$ receptors (Zhou *et al.*, 2011).

Apart from the recruitment of monocytes, several cellular functions of monocytes and macrophages, including the activation status, uptake of LDL and survival, play an important role in atherogenesis (Moore and Tabas, 2011). Human monocytes and macrophages primarily express LPA_1 and LPA_2, and LPA has been shown to mediate the effects of MM-LDL on monocyte activation via LPA_1 (Fueller *et al.*, 2003; D'Aquilio *et al.*, 2007). In agreement with this finding, LPA stimulates the expression of the pro-atherogenic cytokine IL-1β in a GPCR-dependent manner in a murine macrophage cell line (Chang *et al.*, 2008b). Moreover, LPA increases the uptake of ox-LDL in monocytes/macrophages (Llodra *et al.*, 2004; Chang *et al.*, 2008b). In J774A murine macrophages, which express LPA_2 and LPA_3, LPA-induced lipid accumulation is mediated by the up-regulation of the scavenger receptor A and can be inhibited by the LPA_1/LPA_3 receptor antagonist Ki16425 (Chang *et al.*, 2008a). In addition, LPA binds to the nuclear receptor PPARγ and induces PPARγ-dependent CD36 expression in murine macrophages (McIntyre *et al.*, 2003). PPARγ activation in macrophages has anti-inflammatory effects, and conditional deletion of PPARγ in macrophages enhances atherosclerosis (Babaev *et al.*, 2005; Bouhlel *et al.*, 2007). Therefore, an atheroprotective role of LPA by activation of PPARγ could be envisioned, if this mechanism is relevant *in vivo*. Although LPA is known to increase monocyte migration (Zhou *et al.*, 1995; Gustin *et al.*, 2008b), inhibition of reverse transmigration of monocytes by

LPA has been found, which may play a role in the regression of atherosclerosis (Llodra *et al.*, 2004). Moreover, LPA protects macrophages from apoptosis by activating phosphoinositide 3-kinase (PI3K), indicating that LPA impairs the removal of plaque macrophages (Koh *et al.*, 1998).

SMCs accumulate in advanced atherosclerotic plaques, and apoptosis of SMCs is associated with accelerated atherosclerosis. However, exacerbated proliferation of SMCs and the conversion to a pro-inflammatory SMC phenotype may promote the progression of atherosclerosis by enhanced monocyte recruitment (Zeiffer *et al.*, 2004). LPA potently stimulates the proliferation of cultured SMCs and most probably contributes to ox-LDL-induced SMC proliferation (Tokumura *et al.*, 1994; Natarajan *et al.*, 1995; Kim *et al.*, 2006; Damirin *et al.*, 2007; Komachi *et al.*, 2009). LPA mediates the MM–LDL-induced expression of CCL20 in SMCs via LPA receptors, which is secreted from atherosclerotic plaques and elevated in the circulation of patients with hyperlipidaemia (Calvayrac *et al.*, 2011). However, the functional role of LPA-induced CCL20 in atherogenesis needs to be determined. Tissue factor (TF) is a critical determinant of atherosclerotic plaque thrombogenicity. LPA up-regulates TF expression in SMCs via G_i-protein–mediated activation of ERK1/2 (Cui *et al.*, 2003). Furthermore, LPA induces the pro-atherogenic factors CCL2 (MCP-1) and IL-6 in human vascular SMCs *in vitro* (Kaneyuki *et al.*, 2007; Hao *et al.*, 2010).

Taken together, lipoprotein-derived LPA plays a crucial role in atherogenesis by promoting the recruitment of monocytes. Therapeutic targeting of LPA_1 or LPA_3 on endothelial cells may be a promising approach against atherosclerosis.

LPA in vascular remodelling

The arterial vessel wall can adapt to variety of environmental cues, such as changes in pressure, flow, or oxygen supply, by a process called vascular remodelling that mainly includes a response of the SMCs and results in structural alterations of the tunica media (Gibbons and Dzau, 1994). The media may enlarge, for example in arterial or pulmonary hypertension, or diminish, as in the formation of aneurysms. Furthermore, neointimal accummulation of SMCs is characteristic of vascular repair after injury, for example following stent implantation into atherosclerotic arteries (called restenosis). In addition, endothelial cells and inflammatory cells like monocytes/macrophages affect SMC function and the production of extracellular matrix proteins, thereby playing an important role in vascular remodelling (Schober, 2008). The phenotypic switch of SMCs from a contractile, quiescent state towards a synthetic, proliferative state is a common feature during vascular remodelling and may be causally related to neointima formation (Owens *et al.*, 2004).

Both migration and proliferation of medial SMCs following vascular injury have been implicated in neointima formation (Schwartz *et al.*, 1995). *In vitro*, LPA has been shown to induce the proliferation of SMCs via activation of LPA_1 and $G_{i/q}$ proteins and involves PKC, ERK1/2, the PI3K/PKB (Akt) pathway and MAPK cascades (Tokumura *et al.*, 1994; Seewald *et al.*, 1997; 1999; Gennero *et al.*, 1999; Schmitz *et al.*, 2002; Xu *et al.*, 2003; Gouni-Berthold *et al.*, 2004; Baldini *et al.*, 2005; Kim *et al.*, 2006; Komachi *et al.*, 2009). In contrast, combined genetic deletion of LPA_1 and LPA_2 is required to inhibit serum-induced growth of SMCs (Panchatcharam *et al.*, 2008). Furthermore, LPA enhances migratory activity of vascular SMCs by $G_{i/q}$ protein-coupled LPA_1 receptor-mediated activation of the p38MAPK pathway (Kim *et al.*, 2006; Damirin *et al.*, 2007; Komachi *et al.*, 2009; Zhou *et al.*, 2009). However, SMCs from $LPA_1^{-/-}$ mice showed increased migration due to compensatory up-regulation of LPA_3 (Panchatcharam *et al.*, 2008). Whereas the migration of LPA_2-deficient SMCs is not impaired, combined deletion of LPA_1 and LPA_2 inhibits SMC migration (Panchatcharam *et al.*, 2008). These results indicate that LPA_3 also promotes SMC migration in the presence of LPA_2. Neointimal SMCs display a pro-inflammatory phenotype driven by activated NF-κB signalling, which may promote leucocyte recuitment to the injured artery (Zeiffer *et al.*, 2004). LPA increases the expression of IL-6 and CCL2 in vascular SMCs via LPA_1 and PKC-mediated p38 MAPK and NADPH oxidase-dependent generation of reactive oxygen species, respectively (Kaneyuki *et al.*, 2007; Hao *et al.*, 2010). The early growth response gene 1 (Egr1) is crucial for SMC proliferation and neointima formation (Khachigian, 2006). Egr1 is induced by LPA-mediated activation of the transcription factors cAMP response element-binding (CREB) and serum response factor (SRF) in SMCs (Cui *et al.*, 2006). However, the role of LPA-induced Egr1 in vascular remodelling remains to be defined. The reduced expression of contractile proteins is characteristic for synthetic SMCs, and this dedifferentiation process has been implicated in neointima formation (Owens *et al.*, 2004). LPA species with an unsaturated fatty acyl chain selectively down-regulate contractile protein expression in SMCs via sustained activation of ERK1/2 and p38 MAPK (Hayashi *et al.*, 2001). This effect of LPA on SMC dedifferentiation has been attributed to the activation of LPA_3 using LPA receptor-specific antagonists and agonists (Zhou *et al.*, 2010). In agreement with this finding, no differences in LPA-induced SMC dedifferentiation have been observed between wild-type, $LPA_1^{-/-}$, $LPA_2^{-/-}$, $LPA_1^{-/-}/LPA_2^{-/-}$ and $PPAR\gamma^{-/-}$ SMCs (Guo *et al.*, 2008; Panchatcharam *et al.*, 2008). However, the PPARγ agonist rosiglitazone reduced the expression of SMC markers, like unsaturated LPA, indicating different pathways for PPARγ- and LPA-induced SMC differentiation (Zhang *et al.*, 2004). Taken together, LPA acts in multiple ways on SMCs *in vitro*, which suggests that LPA promotes vascular remodelling and neointima formation.

To study the role of LPA in vascular remodelling *in vivo*, short-term incubation of the carotid arteries with LPA has been performed. Intriguingly, transient treatment only with unsaturated LPAs, like LPA18:1, LPA20:4 or the LPA analogue 1-AGP18:1 (1-O-octadecenyl glycerophosphate), triggers the formation within weeks of a neointima that primarily consists of SMCs (Yoshida *et al.*, 2003; Zhang *et al.*, 2004; Cheng *et al.*, 2009; Subramanian *et al.*, 2010). Blocking LPA18:1-induced ERK and p38MAPK activation prevented neointima formation (Yoshida *et al.*, 2003). Furthermore, LPA20:4 and 1-AGP18:1-induced neointima formation was reduced by treatment with a PPARγ antagonist and in mice with PPARγ-deficiency in vascular wall cells (Zhang *et al.*, 2004; Cheng *et al.*, 2009). Although LPA20:4-induced neointima formation was partially inhibited by pertussis toxin and the LPA_1/LPA_3

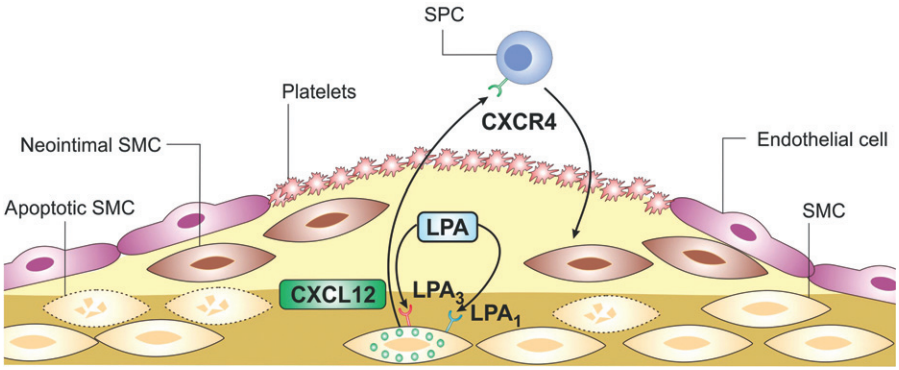

Figure 4

LPA induces neointima formation after vascular injury. Following vascular injury, activated platelets adhere to the denuded surface of the vessel wall and medial SMCs undergo apoptosis. These early events after vascular injury may induce the production of LPA, which increases CXCL12 in the vessel wall through its receptors LPA_1 and LPA_3. CXCL12 is released into the circulation and recruits SPC via its receptor CXCR4 to the injury site. These SPCs differentiate into neointimal SMCs, which form the neointimal lesion.

antagonist dioctylglycerol pyrophosphate in rats, which do not express LPA_3 in the carotid wall, neointimal growth following 1-AGP18:1 incubation was not impaired in $LPA_1^{-/-}$, $LPA_2^{-/-}$ and $LPA_1^{-/-}/LPA_2^{-/-}$ mice (Zhang *et al.*, 2004; Cheng *et al.*, 2009). Neointima formation by carotid ligation is increased in $LPA_1^{-/-}$ mice, whereas a combined deficiency of LPA_1 and LPA_2 diminishes neointimal growth (Panchatcharam *et al.*, 2008). The compensatory overexpression of LPA_3 in SMCs from $LPA_1^{-/-}$ mice, which is absent in $LPA_1^{-/-}/LPA_2^{-/-}$ SMCs, is associated with enhanced neointima formation (Panchatcharam *et al.*, 2008). PPARγ activation in SMCs has been shown to inhibit proliferation and migration and attenuates neointimal hyperplasia after vascular injury (Lim *et al.*, 2006; Lee *et al.*, 2009; Zhang *et al.*, 2011). Accordingly, 1-AGP18:1, but not rosiglitazone, increases injury-induced neointima formation, indicating that LPA-mediated PPARγ activation does not play a role in vascular remodelling following vascular injury (Cheng *et al.*, 2009). Taken together, these results show that molecular mechanisms of LPA-dependent neointima formation may differ between local treatment with LPA and in established animal models of vascular remodelling.

In atherosclerosis-prone $Apoe^{-/-}$ mice, treatment with the LPA_1/LPA_3 antagonist Ki16425 diminished neointimal hyperplasia after carotid wire injury by reducing the SMC and macrophage content in the lesions, indicating that LPA promotes injury-induced neointima formation via LPA receptors (Subramanian *et al.*, 2010). Interestingly, unsaturated but not saturated LPA induces sustained up-regulation of the chemokine CXCL12 (stromal cell-derived factor 1) in the vessel wall, which mobilizes and recruits smooth muscle progenitor cells (SPCs) from the bone marrow into the injured artery (Schober, 2008; Subramanian *et al.*, 2010) (Figure 4). Silencing of either LPA_1 or LPA_3 in the vessel wall impaired the LPA20:4-induced neointima formation and SPC mobilization, suggesting that, similar to atherogenic monocyte recruitment, these two LPA receptors independently trigger the vascular response (Subramanian *et al.*, 2010). It remains to be determined whether this functional independency might be due to the formation of heterodimers between LPA_1 and LPA_3, and whether LPA_1/LPA_3 heterodimers have an increased binding affinity to unsaturated LPAs (Zaslavsky *et al.*, 2006). The different mechanisms that were observed in LPA-induced neointimal hyperplasia might be dose-dependent. Whereas 40 μM of LPA20:4 has been found to trigger CXCL12 expression, unsaturated LPAs in a dosage range between 1 and 10 μM induce ERK1/2/p38MAPK- or PPARγ–dependent neointima formation (Yoshida *et al.*, 2003; Zhang *et al.*, 2004; Cheng *et al.*, 2009; Subramanian *et al.*, 2010). However, the fact that pharmacological inhibition of LPA_1 and LPA_3 after carotid injury inhibits CXCL12 expression and impairs CXCL12-dependent SPC mobilization indicates that the 40 μM LPA20:4 dose used for local carotid treatment closely mirrors the role of LPA after vascular injury (Subramanian *et al.*, 2010). Microvesicles released from apoptotic SMCs following injury have been found to up-regulate CXCL12 expression in non-injured SMCs, and blocking the apoptosis of SMCs following vascular injury greatly inhibits CXCL12 expression (Zernecke *et al.*, 2005). Taking into account the role of microvesicles in LPA generation (Fourcade *et al.*, 1995), increased LPA production due to the release of microvesicles from apoptotic SMCs may contribute to the up-regulation of CXCL12 in the injured arteries. In addition, activated platelets adhering to the denudated vascular surface may not only present CXCL12 during the recruitment of SPCs, but also increase CXCL12 expression by enhancing LPA formation (Zernecke *et al.*, 2005).

Remodelling of the pulmonary vasculature occurs during the hypoxia-driven development of pulmonary hypertension and is characterized by the thickening of the media and adventitia in pulmonary arteries and muscularization of non-muscular alveolar wall vessels (Stenmark *et al.*, 2006). Interestingly, hypoxia-induced pulmonary hypertension and remodelling is accelerated in both $ATX^{+/-}$ mice and $LPA_1^{-/-}/LPA_2^{-/-}$ mice (Cheng *et al.*, 2012). In $LPA_1^{-/-}/LPA_2^{-/-}$ mice, thickening of the arteriolar vessel wall is already evident during normoxia, and aging results in severe pulmonary hypertension (Cheng *et al.*, 2012). This increased remodelling of pulmonary vessels was associated with increased expression of genes involved in endothelin-1 signalling (Cheng

et al., 2012). In contrast, the deficiency of either LPA_1 or LPA_2 is not associated with pulmonary hypertension. This indicates that LPA signalling via LPA_1 and LPA_2 in the pulmonary vasculature has an important homeostatic role and appears to have protective effects in hypoxia-induced pulmonary hypertension.

Conclusions and future directions

LPA has atherogenic actions, and specific LPA receptors have been identified that may mediate some of these harmful LPA activities. The experimental evidence is based not only on numerous *in vitro* studies, but also on several *in vivo* studies. LPA does not only enhance atherosclerosis, but also *vice versa*, cardiovascular risk factors affect the ATX/LPA axis thereby further aggravating the progression of atherosclerotic diseases. Obesity and hyperlipidaemia enhance ATX expression and activity, and the generation of LPA in the circulation. Increased ATX-dependent LPA formation from LDL-derived LPC promotes endothelial chemokine (CXCL1) secretion leading to the recruitment of monocytes to the atherosclerotic lesion, and thereby enhancing atherosclerosis (Figure 3). Endothelial $LPA_{1/3}$ receptors and Rho-kinase are critical here. LPA also increases the uptake of ox-LDL in monocytes/macrophages by up-regulation of the scavenger receptor A, and inhibits monocyte egression from plaques. LPA may also induce these effects by binding to $LPA_{1/3}$ and to the nuclear receptor PPARγ. LPA also stimulates VSMC dedifferentiation, proliferation and neointima formation. Also here, LPA_1 and LPA_3 receptors as well as PPARγ seem to be involved; the situation is, however, complex, as the mechanism of LPA-dependent neointima formation seems to differ between local treatment with LPA and established animal models of vascular remodelling. Lastly but not least, LPA accumulating in the lipid-rich core may contribute to acute thrombus formation after plaque rupture by stimulation of the platelet LPA_5 receptor and Rho-kinase (Figures 1, 2). Future studies might be directed to explore the role of the non-Edg receptor family of LPA receptors in various animal models of atherosclerosis, to determine the expression of individual LPA receptors in human atherosclerotic lesions *in situ* and to evaluate the effects of novel LPA receptor antagonists on the development, progression and regression of atherosclerosis in animal models, as well as on thrombosis induced by atherosclerotic plaques in human models. Clinical studies could determine whether plasma levels of defined LPA molecular species could be a novel biomarker of cardiovascular risk. In such studies, the addition of pharmacological ATX inhibitors to the blood collection tube might be important to minimize artificial *in vitro* LPA formation during blood handling.

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Statement of conflict of interest

The authors have no conflict of interest.

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